MYCOTAXON

ISSN (print) 0093-4666 (online) 2154-8889 © 2016. Mycotaxon, Ltd.

January–March 2016—Volume 131, pp. 87–94

http://dx.doi.org/10.5248/131.87

Taxonomic re-evaluation and phylogenetic position of *Hemibeltrania cinnamomi* within *Xylariales*

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Abstract — *Hemibeltrania cinnamomi* associated with leaf blotch disease of *Cinnamomum malabatrum* (*Lauraceae*) is described and illustrated. Phylogenetic LSU and ITS sequence analyses support the fungus as a species of *Hemibeltrania* associated with other *Beltrania*-like genera. A synoptic table of *Hemibeltrania* species is provided.

Key words — asexual *Ascomycetes*, plant pathogen

Introduction

When revisiting *Beltrania* and related genera, Pirozynski (1963) introduced the genus *Hemibeltrania* (typified by *H. cinnamomi*) for a causal fungus of cinnamon leaf spot in Sierra Leone characterized by conidia described as ellipsoid to obovate and 12–16 × 10–12 µm. The phylogenetic placement of *Hemibeltrania* is unknown.

In December 2014, a survey was conducted to investigate microfungal diversity in the natural forests of Kaladi and the surrounding area within the southern Western Ghats, India. An unusual species of *Hemibeltrania* was discovered, which causes foliar blotches in *Cinnamomum malabatrum*. The present study aims to authenticate the species of *Hemibeltrania* using morphological characters and resolve the phylogenetic position of the genus using ITS and LSU sequence data.

Materials & methods

Isolates and morphology—Conidia were directly isolated from the surface of a leaf spot and observed under a Nikon stereomicroscope (Model SMZ–1500 with Digi-CAM). Single conidial cultures were established on 2% Potato Dextrose Agar (PDA) and 2% Malt Extract Agar (MEA) plates (Crous et al. 2009). For morphotaxonomic studies and photomicrographs a Carl Zeiss Image Analyzer 2 microscope was used. Conidia and conidiophores were mounted in lactic acid cotton blue and measured using AxioVision REL.4.8 software, with 30 observations per structure. Colony characteristics in culture were studied on MEA and PDA (Crous et al. 2009). Herbarium specimens were deposited in Ajrekar Mycological Herbarium (AMH 9667), and the culture was accessioned and preserved in the National Fungal Culture Collection of India (NFCCI-3695), Agharkar Research Institute, Pune, India.

DNA extraction, amplification, and phylogenetic analyses—Colonies of *H. cinnamomi* were grown on MEA plates, and genomic DNA was extracted following the rapid salt-extraction method of Aljanabi & Martinez (1997). Fragments containing the region encoding the 28S nrDNA (LSU) and ITS 1-5.8S nrDNA-ITS 2 (ITS) were amplified using primer pairs LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for LSU and primer pairs ITS4 and ITS5 (White et al. 1990) for ITS. PCR conditions, sequence alignments, and phylogenetic analyses followed the protocols of Crous et al. (2006). Thirty LSU sequences of the order *Xylariales* and an outgroup of two sequences (*Trichoderma* and *Lulworthia*) were downloaded from GenBank. Twenty-seven ITS sequences of the order *Xylariales* and an outgroup of two sequences (*Trichoderma* and *Lulworthia*) were downloaded also for phylogenetic analyses. The ITS and LSU regions were analysed by the Maximum Parsimony method with bootstrap (BS) analysis using 1000 replications in MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0). Sequence data were deposited in GenBank. Family placement of the sequences follows Shirouzu et al. (2010) and Hidayat et al. (2014).

Taxonomy

Hemibeltrania cinnamomi (Deighton) Piroz., Mycol. Pap. 90: 32 (1963) Figs 1, 2

Symptoms: necrotic, reddish brown to brick brown, amphigenous, irregular.

On the natural substrate: Conidiophores distinct, single or slightly branched toward the apex, erect, straight or flexuous, cylindrical, slightly sinuate, 4–8-septate, smooth, dark brown at the base, pale brown toward the apex, thin-walled, 130–250 × 3.5–6 µm, arising from a radially lobed basal cell, 10–11.5 µm diam. Conidiogenous cells mono- and polyblastic, integrated or discrete, terminal, determinate or sympodial extended, denticulate; denticles cylindrical. Conidial secession schizolytic. Conidia solitary, limoniform, broadly ellipsoidal or obovate to irregular, rounded or obtuse at the apex, sometimes attenuate, subulate or rounded at the base with a small frill at the base, acrogenous becoming acropleurogenous after several sympodial extensions, unicellular, smooth, 12–25 × 8–13 µm, hyaline, subhyaline to pale olivaceous.

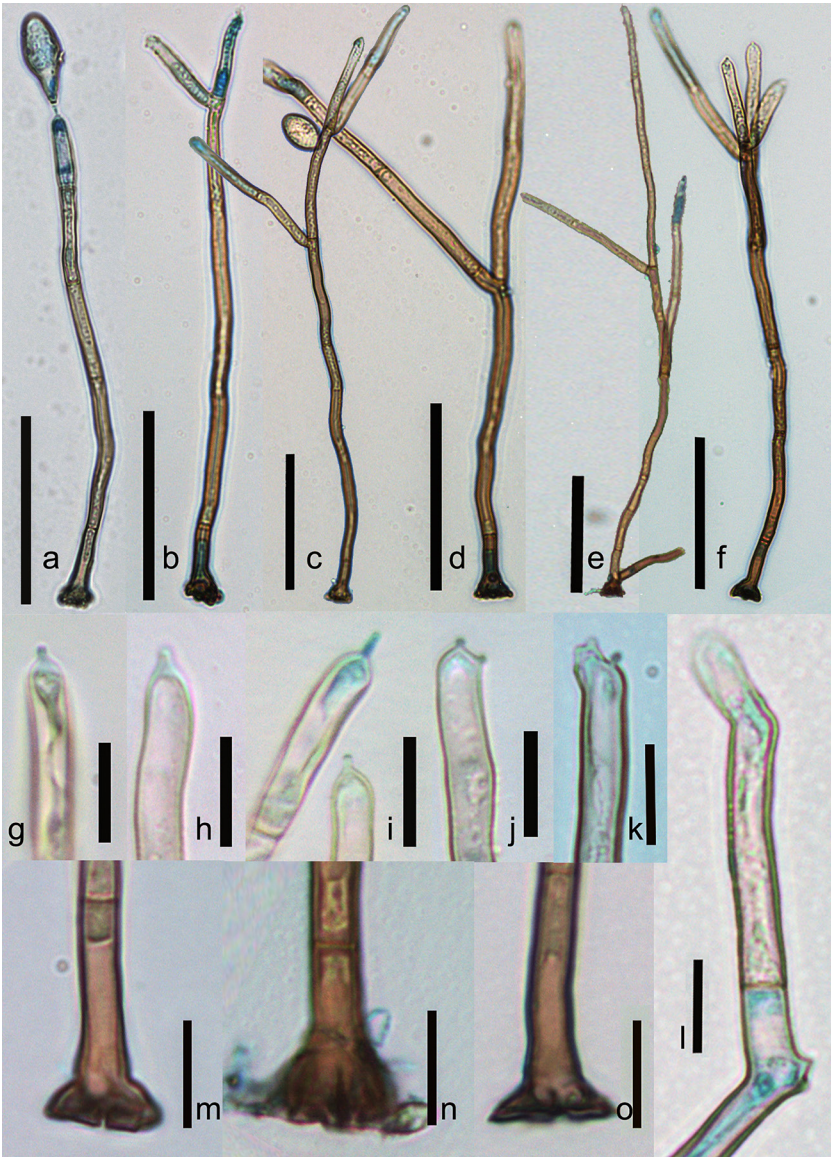

Fig. 1. *Hemibeltrania cinnamomi* (AMH 9667).
a–f: Conidiophore branching. g–l: Denticulate conidiogenous cells. m–o: Conidiophore bases.
Bars: a–f = 50 µm; g–o = 10 µm.

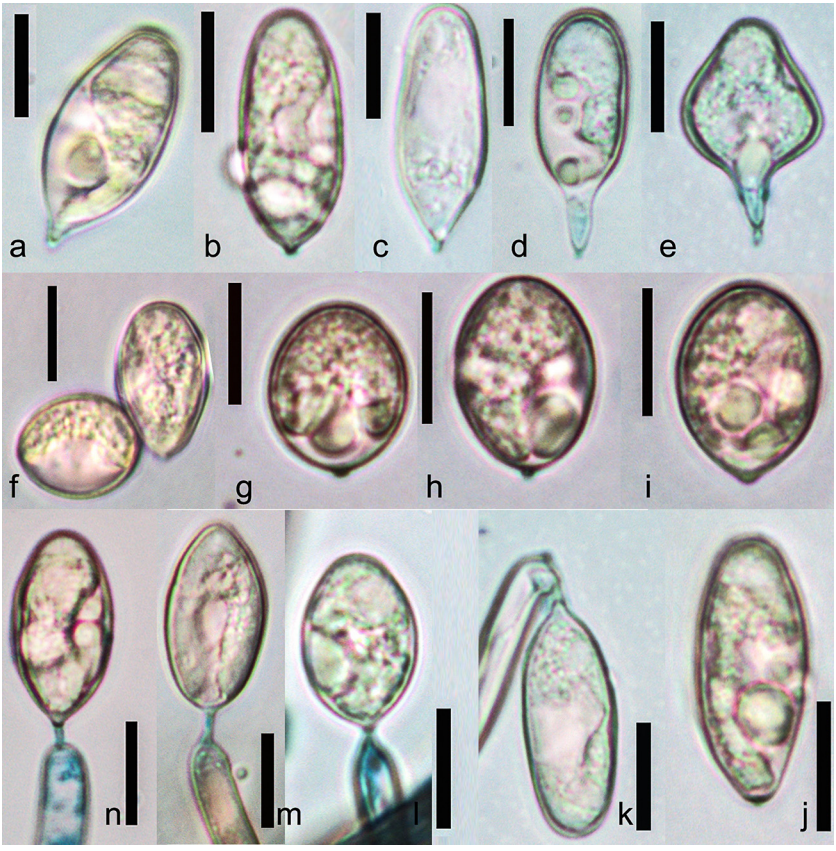

Fig. 2. *Hemibeltrania cinnamomi* (AMH 9667).
a–j: Conidia. k–n: Conidial attachments. Bars: 10 µm.

Colonies on MEA slow to moderate growing, 35–45 mm diam after 7 days, grayish or mouse gray, later turning dark gray, velutinous, margin irregular, reverse blackish to blackish blue. Conidia 12–17 × 9.5–12 µm.

Specimen examined: **INDIA**. **Kaladi**: **Western Ghats**, Kerala, 10°16′N 76°43′E, on leaves of Cinnamomum malabatrum (Burm. f.) J. Presl (*Lauraceae*) December 2014, K.C. Rajeshkumar & K. Madhusudhanan (AMH 9667; culture NFCCI 3695; Genbank KT119564, KT119565).

Phylogenetic analyses

LSU (KT119565): based on MegaBLAST search of GenBank LSU sequences, the closest hits are: *Xylaria* sp. (JQ862647.1, identities = 96%, gaps 0.2%);

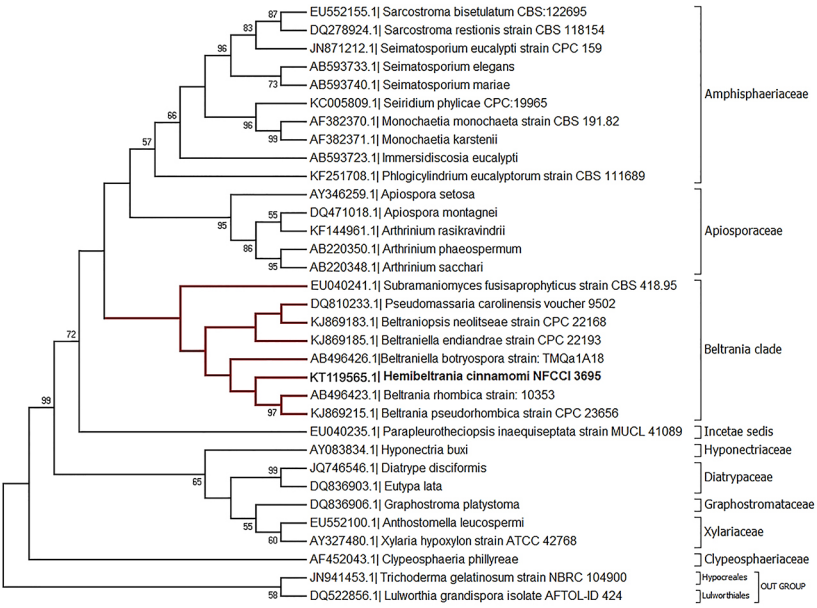

Fig. 3. LSU phylogenetic tree generated from Maximum Parsimony (MP) analyses of the aligned sequences of *Hemibeltrania cinnamomi* and allied taxa of the order *Xylariales*, with *Trichoderma* and *Lulworthia* as outgroup. Percentage bootstrap support (≥50%) is shown on the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The bootstrap test was conducted with 1000 replicates.

Xylaria sp. (JQ862650.1, identities = 96%, gaps = 0.2%); and *Xylaria badia* (JQ862643.1, identities = 96%, gaps = 0.2%).

ITS (KT119564): A MegaBLAST of the ITS sequence showed that the closest hits are: *Hemibeltrania* sp. CL12WA (JQ621881.1, identities = 99%, gaps 0.4%); *Beltraniella endiandrae* CPC 22193 (KJ869128.1, identities = 93%, gaps = 3%); and *Beltrania pseudorhombica* CPC 23656 (KJ869158.1, identities = 92%, gaps = 4%).

The ITS and LSU phylogenetic analyses supported the genus *Hemibeltrania* as closely related to *Beltrania*, *Beltraniopsis*, *Beltraniella*, and *Subramaniomyces* and forming a unique monophyletic lineage in the *Xylariales* and closely allied to *Amphisphaeriaceae* and/or *Apiosporaceae* (Figs 3, 4).

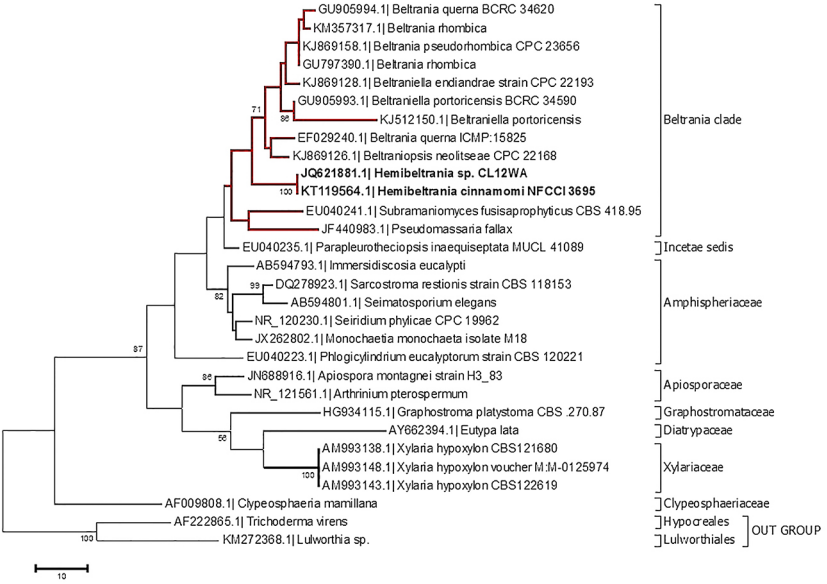

Fig. 4. ITS phylogenetic tree generated from Maximum Parsimony (MP) analyses of the aligned sequences of *Hemibeltrania cinnamomi* and allied taxa, with *Trichoderma* and *Lulworthia* as outgroup. Percentage bootstrap support (≥50%) is shown on the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The bootstrap test has 1000 replicates.

Discussion

Hemibeltrania comprises 12 accepted species (MycoBank 2015; Table 1). Taxonomic study of the present collection revealed that in *H. cinnamomi,* conidial shapes and sizes are more variable than the original description: conidia are longer than 10 µm, while conidial shapes range from limoniform, obovate, and broadly ellipsoid to irregular. The symptomatology and other taxonomic characters, however, are identical to the type description given by Pirozynski (1963). Considering the variable conidial shape and size observed in AMH 9667, we propose a broad species concept for *H. cinnamomi* that might be expanded in the future based on re-examination and phylogenetic analysis of *H. nectandrae*, which shows some affinity with *H. cinnamomi*.

Earlier studies have shown that *Beltrania* and related genera (*Beltraniella*, *Beltraniopsis*, *Subramaniomyces*) are phylogenetically allied with *Amphisphaeriacaeae*, *Xylariales* (Shirouzu et al. 2010, Hidayat et al. 2014). Shirouzu et al. (2010) also noted that *Beltraniella* anamorphs and

Table 1. Comparative morphology of accepted *Hemibeltrania* species

Species	Conidia		Conidiophore size (µm)
	Shape	Size (µm)	
H. cinnamomi	Obovoid/broadly ellipsoid/limoniform	12–25 × 8–13	130–250 × 3.5–6
H. convallariae	Ovoid	14–24 × 10–15	≤2000 × 13–23
H. cymbiformis	Cymbiform	21–24 × 3–4.5	≤140 × 2.2–4.5
H. decorosa	Fusiform	17–22 × 3.5	≤250 × 4–6.5
H. echinulata	Ovoid	12–14 × 5.5–7	140–300 × 3.5–5
H. laxa	Obclavate/navicular	13–17 × 2–3	50–350 × 3–7
H. malaysiana	Ellipsoid/navicular	13–20 × 3–5	≤400 × 4–7
H. mitrata	Mitriform	14–17 × 4.5–5.5	35–85 × 3–4.5
H. nectandrae	Limoniform	15–23 × 10–13	≤250 × 4–6
H. ovalispora	Obovoid	12–14 × 7	≥750 × 4–6
H. saikawae	Clavate/cylindrical	14–19 × 2.5–3	10–155 × 2–3
H. urbanodendri	Ovoid/obovoid/ biconic/limoniform	12–25 × 6–10	55–106 × 4–6

Pseudomassaria teleomorphs are phylogenetically linked. Our phylogenetic analysis of *H. cinnamomi* does support *Hemibeltrania* within the *Xylariales*. However, the family in which it should be placed remains ambiguous.

Acknowledgements

We are indebted to Dr. Elaine Malosso (Federal University of Pernambuco, Brazil) and Dr. De-Wei Li (The Connecticut Agricultural Experiment Station Valley Laboratory, USA) for reviewing this manuscript. Thanks are also due to Department of Science and Technology (DST), Government of India, New Delhi, for providing financial support and the Director, ARI, for providing facilities. RFCR is grateful to the Cuban Ministry of Agriculture and "Programa de Salud Animal y Vegetal" (project P131LH003033) for facilities. We acknowledge the assistance provided by Dr. P.M. Kirk and Drs. V. Robert and G. Stegehuis through the Index Fungorum and MycoBank websites. Dr. Lorelei L. Norvell's editorial and Dr. Shaun Pennycook's nomenclatural reviews are greatly appreciated.

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